CODING FORM FOR SRC INDEXING

Microfiche No.

OTS0538189

New Doc I.D.

86-930000209S

Old Doc I.D.

Date Produced

3/30/93

Date Recieved

5/04/93

TSCA section

8D

Submitting Organization

E I DU PONT DE NEMOURS & CO

Contractor

Document Title

METABOLISM AND DERMAL PENETRATION OF [] BY LABORATORY RATS

Chemical Category

Section 8d

86930000209 S

FOR DUPONT USE ONLY

HLR 166-93

Study Title

27pp

Metabolism and Dermal Penetration of [CBI] by the Laboratory Rat

Project Title

Studies of the Effects of [CBI] on Cell Proliferation and DNA Damage and Repair in Urinary Bladder, and on the Induction of Methemoglobin in Rats. Development of Analytical Techniques for Biomonitoring and Studies of the Dermal Penetration and Toxicokinetics of [CBI]

Author

Stephen G. Hundley
Senior Research Biochem.

Study Completed on

March 30, 1993

Performing Laboratory

E. I. du Pont de Nemours and Company
Haskell Laboratory for Toxicology and Industrial Medicine
Elkton Road, P.O. Box 50
Newark, Delaware 19714

Sponsor

E. I. du Pont de Nemours and Company
DuPont Chemicals
Wilmington, Delaware

Laboratory Project ID

[CBI]
HLR 166-93

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STUDY INFORMATION

Material Tested:

1. [CBI]
2. [CBI]

Purity: 1) 99.5%
2) >98%

Synonyms: [CBI]

CAS Registry Number: [CBI]

Sponsor: E.I. du Pont de Nemours and Co., Inc.
DuPont Chemicals
Wilmington, Delaware

Study Initiated/Completed:

10/24/91 - 2/1/93

SUMMARY

Male rats were dosed orally by gavage with 1 or 500 mg [CBI]/kg body weight or were exposed dermally for six hours to 1 mg [CBI] Urine and feces were collected for a period of 48 hours following the oral gavage doses and for 24 hours following dermal application. A complete organ and tissue distribution of radioactivity was conducted for the dermal application group.

Approximately 70 percent of the 1 or 500 mg/kg oral dose was excreted in urine by 48 hours after dosing. However, 60 percent of the 1 mg/kg dose was excreted in urine during the initial 12 hour post-dosing time interval compared to 18 percent of the 500 mg/kg dose. Approximately 14 percent of dermally applied [CBI] was absorbed into systemic circulation during a six-hour application. A substantial amount of the dermally applied dose (77 percent) evaporated from the application site. Urinary excretion represented 88 percent of the absorbed dose with approximately 63 percent of the absorbed dose being excreted in the 0-to 12-hour post-application urine sample.

Two major urinary metabolites identified in the 1 mg/kg oral dose and the 1 mg dermal application dose were [CBI] and [CBI]. Both compounds appear in substantial amounts following glucuronidase/sulfatase enzyme hydrolysis of the urine samples. In the 12-hour urine sample from the 1 mg/kg orally dosed rats and the 1 mg dermally dosed rats, [CBI] represented 33 and 27 percent of the oral or dermally absorbed dose, respectively. Similarly, [CBI] represented approximately 19 and 16 percent. Either metabolite is present in urine in sufficient quantities to provide an accurate assessment of internal [CBI] exposure under workplace conditions.

SIGNATURES

Written by: Stephen Hundley 4/6/93

Stephen G. Hundley
Senior Research Biochemist
Chronic, Biochemical and
Inhalation Toxicology

Approved by: Robert W Rickard 4/7/93

Robert W. Rickard
Manager
Chronic, Biochemical and
Inhalation Toxicology

ACKNOWLEDGMENTS

The following individuals are acknowledged for their fine research efforts on this project: Terry Greer, Walter Moll, Darlene Ferster, Jim Williamson, Paul Lieder and Mary Boyd. Chris Shoop and Susan Murray are acknowledged for manuscript preparation.

INTRODUCTION

Workplace exposure to [redacted] is currently assessed by air-monitoring techniques and by a non-compound specific measure of methemoglobinemia. However, air-monitoring cannot measure potential dermal contact or exposure and surface monitoring may not reflect actual employee exposure. A specific urinary biological monitor can be used to estimate the extent of exposure to [redacted]

The present study was designed to characterize the urinary metabolites of [redacted] and to determine the relative distribution of these metabolites. In addition, the percent of an administered [redacted] dose excreted in urine was determined. Dermal absorption of [redacted] was evaluated as well as metabolite excretion following dermal absorption. The laboratory rat was used to determine each of these parameters.

MATERIAL AND METHODS

Animals and Husbandry

Male Crl:CD®BR rats (Charles River Breeding Laboratories, Raleigh, NC) were selected and cared for under the following conditions:

- o Age: 6-9 weeks upon arrival
- o Housing: One week quarantine upon arrival in a controlled temperature (23 ± 2C°), humidity (40-60% relative humidity), and light cycle (12-hour light-dark cycle) environment.
- o Diet: Purina Certified Laboratory Chow® #5002 and water ad libitum. Feed and water were periodically assayed for

chemical contaminants; no contaminants that would interfere with results of this study were detected.

o Identification: Each rat was given an individual number on a cage card upon arrival. The numbers were maintained during the course of the study.

Animals were received and quarantined by the Haskell Laboratory Animal Resources Group according to their standard operating procedure. Rats were selected for study after the quarantine period elapsed, based upon weight uniformity (an approximate 10 gram weight range for each dosing group). Only healthy animals were released from quarantine.

Chemicals

The following HPLC-grade solvents were purchased from E. M. Science; acetonitrile, methanol and water (for HPLC analysis). Reagent grade chloroform and glycerol were purchased from Baker Scientific. A.C.S. grade ammonium acetate, ethanol, hydrochloric acid and sodium hydroxide were purchased from Fisher Scientific. Heparin sodium was purchased from Upjohn, Co.

Test Compound

The [CBI] purchased from Amersham (Arlington Heights, IL) was >98% pure with a specific radioactivity content of approximately 18.5 mCi/mmole. Non-labelled [CBI] was supplied by DuPont Chemicals as a clear faint yellow liquid with a purity of 99.5%. Radiochemical purity of [CBI] in a dosing formulation was evaluated by HPLC/radioactivity-flow monitoring (Figure 1).

Dosing Routines

Male rats (4 in each dosing routine) received a single 500 mg [CBI]/kg body weight oral-gavage dose; a single 1 mg [CBI]/kg body weight oral-gavage dose; or a dermal application of 1 mg [CBI]. The 500 mg/kg dose consisted of a mixture of corn oil and [CBI] (4.4 mL corn oil to 0.625 mL [CBI]; 1 mL of this mixture delivered a 500 mg/kg dose to a 250 g rat. Non-labelled [C] was mixed with [CBI] such that each rat received 30.5 µCi of ^{14}C-radioactivity with a specific radioactivity content of 0.244 µCi/mg.

The 1 mg/kg dose was prepared in a 9:1 (corn oil:methanol) dosing vehicle such that 1 mL delivered a 1 mg/kg dose to a 250 g rat. Each rat received 21.9 µCi of ^{14}C-radioactivity with a specific radioactivity content of 86.5 µCi/mg.

The dermal dose was formulated in a 10:20:70 (ethanol:glycerol:water) matrix such that 100 µL contained 1 mg of [CBI] Non-labelled [C] was mixed with [CBI] such that each rat received 21.6 µCi of ^{14}C-radioactivity with a specific radioactivity content of 21.6 µCi/mg.

Metabolism Units and Sample Collections

Each rat on study was placed into a "Roth"-type glass metabolism unit (designed to separate urine from feces), immediately after receiving the radioactive dose. Rats were maintained ad libitum on ground rodent chow and water. Urine and feces were collected from each rat 12, 24, and 48 hours after the oral doses and 12 and 24 hours after dermal application. Tissue samples were not taken from the orally dosed rats at sacrifice nor were fecal samples assayed for radioactivity.

Dermal Application Procedure

Dermally exposed rats had jugular-vein cannulas surgically inserted the day prior to the dermal exposure. On the day of exposure the application site on the back of the animal between the shoulder blades was shaved. A foam rubber protective device was secured in place over the application site with a surgical adhesive. The dose mixture was applied in a 2 x 3 cm area within the shaved area. A metal (perforated) protective cover with non-occlusive gauze was then placed over the foam rubber protective device and secured in place. Six hours following application the metal cover and gauze were removed and the application site was washed. The washing (cleansing) consisted of five cycles of rubbing 5% aqueous Micro® over the application site with a cotton swab and removing the wash with a dry cotton swab. Swabs were placed in the vials for subsequent assay for radioactivity.

Blood samples were drawn through the cannula 1, 2, 3, 4, 6, and 12 hours following dermal application. Approximately 0.2 to 0.3 mL of whole blood was drawn at each timepoint.

Dermally exposed rats were sacrificed by chloroform exposure 24 hours following dermal application. Blood was drawn by cardiac puncture at sacrifice. The following tissues and organs were excised: heart, lungs, liver, spleen, kidneys, testes, brain, G.I. tract (and contents), skin (application site), non-application site skin, and fat. The remaining carcass was saved and the metabolism units washed with Micro®, acetone and water. The cage wash was collected in Nalgene® bottles. All samples from the dermally exposed rats were assayed for radioactivity.

Assessment of o-Toluidine Volatility

Approximately 100 µl of the dermal application dose was transferred to the bottom of a 25-mL capacity glass impinger. A second impinger containing approximately 10 mL methanol was connected to the outlet of the first impinger with a Glenco® fitting. The methanol-containing impinger was attached with tygon tubing to a house-vacuum line. Gentle air flow was then generated through the impinger set-up by house-vacuum for about 45 minutes. Methanol in the second impinger was then assayed for radioactivity. Results indicated approximately 93 percent of the [CBI] evaporated from the first impinger and was trapped by the methanol in the second impinger.

Radioactivity Analysis

Liquid scintillation counting was conducted with Mark III Model 6882 Liquid Scintillation Counters (TM Analytic, Elk Grove, IL). Samples directly assayed (following additions of 12-15 mL scintillation cocktail) included:

- Urine
- Dose solution or dilutions
- Cage washes
- HPLC eluant fractions

Aquasol®-II scintillation cocktail (Du Pont-New England Nuclear, Boston, MA) was used for analysis.

Representative samples of blood, organs, tissues, carcasses and feces were assayed for radioactivity by combustion with Packard Model 306 Tissue Oxidizers (Packard Instrument Co., Downers Grove, IL).

Urinalysis

Urine samples were thawed and a portion transferred to separate vials and adjusted to pH 6 with 1N hydrochloric acid. One mL samples were then incubated overnight at 37°C after addition of a glucuronidase/sulfatase enzyme preparation (B-glucuronidase, type H-1 isolated from Helix pometia and purchased from Sigma Chemical Co.). Companion urine samples were also incubated as non-enzyme treated controls.

All urine samples were prepared for HPLC analysis by mixing 0.5 mL methanol with 0.5 mL of the enzyme treated or non-enzyme treated urine. These samples were centrifuged for 5 minutes with an Eppendorf microfuge to remove particulates. The liquid layer was then passed through a 0.45 µ or 0.22 µ Acrodisc® filter (Gelman Science) into HPLC sample vials (crimp top) and subsequently assayed by HPLC.

HPLC Analysis and Metabolite Purification

HPLC Analyses were conducted with a Perkin-Elmer Series 4 Liquid Chromatograph (Perkin-Elmer, Norvalk, CT). Radioactivity was monitored and the relative percent of each radioactive peak was determined with a Ramona-D radioactive flow monitor (Raytest U.S.A., Inc., Pittsburgh, PA).

The following HPLC conditions were used with the previously mentioned instruments for analysis of reconstituted fecal extracts and urine samples:

1. Columns:
   a) C-8 Adsorbosphere® guard column 21.5 mm
   b) Zorbax® C-8, 21.5 mm id x 25 cm preparative column

2. Solvent Flow Rate: 5.0 mL/min

3. Column Temperature: Ambient

4. Solvents:
   a) Acetonitrile
   b) 0.1 M ammonium acetate adjusted to pH 3.3 with concentrated HCl

5. Gradient System:
   1) 10:90 Acetonitrile:0.1 M ammonium acetate isocratically for 10 min.
   2) 10:90 to 50:50 Acetonitrile:0.1 M ammonium acetate over a 25 min. linear gradient
   3) 50:50 Acetonitrile:0.1 M ammonium acetate isocratically for 15 min.
   4) 50:50 to 10:90 Acetonitrile:0.1 M ammonium acetate linear gradient for 10 min.
   5) 10:90 Acetonitrile:0.1 M ammonium acetate isocratically for 10 min. prior to next injection

HPLC fractions were assayed for radioactivity content by using the Ramona radioactivity chromatogram profile to identify fractions for direct analysis. Samples ranging from 10 to 50 µL were assayed directly by scintillation counting. Those fractions containing radioactivity that corresponded to the radioactive peaks, identified by the Ramona chromatograms, were saved and stored in a freezer (at approximately -20°C). Subsequently each metabolite fraction was taken to dryness under a gentle N_2 stream and stored dry at approximately -20°C until submitted for mass spectral analysis.

RESULTS

Urinary Excretion Following Oral Dosing

Urinary percent of dose values for the 1 and 500 mg/kg oral doses of [CBI] are presented in Table 1. The total percent excreted in urine through 48 hours was similar for the 1 and 500 mg/kg dose (approximately 70 and 69 percent, respectively). However, the 0- to 12-hour average percent of dose values from the 1 mg/kg group were much greater than the 500 mg/kg group (60 percent compared to 18 percent). The percent of dose excreted in the 12- to 24-hour urine sample from the 1 mg/kg dose group represented approximately 9 percent of the administered dose.

Distribution of Radioactivity Following Dermal Application

Table 2 summarizes the distribution of dermally applied [CBI] (1 mg per rat). Approximately 14 percent of the applied dose was absorbed, whereas, only 8 percent of the applied dose was accounted for as non-absorbed radioactivity. The remainder of the applied dose was not accounted for. In separate experiments, [CBI] evaporated in less than one hour under conditions similar to the dermal exposure (see Methods).

Approximately 88 percent of dermally absorbed [CBI] was excreted in the urine by the 24-hour post-application sacrifice. Almost 63 percent was excreted in the 0- to 12-hour post-application urine sample. The tissues and carcass contained approximately 7 percent of the absorbed dose at the 24-hour post-application sacrifice.

Tissue Distribution Following Dermal Application

The radioactivity concentrations in organs and tissues are presented as µg equivalents of [CBI] per gram tissues (Table 3). Skin (distal from the site of dermal application) contained the highest concentration of radioactivity, followed by liver concentrations (0.138 and 0.071 µg equivalents per gram, respectively). Whole blood concentrations averaged 0.017 µg equivalents per mL, whereas, plasma concentrations averaged 0.007 µg equivalents per mL.

HPLC/Radioactivity Flow Detection Analysis of Urine Samples

Radioactive metabolite profiles in urine were characterized by HPLC/radioactivity-flow analysis (Figures 2-4). Urine from rats dosed at the 500 mg/kg level contained substantial concentrations of unchanged [CBI]. Overnight incubation with glucuronidase/sulfatase enzymes modified the metabolite profile resulting in the appearance of a radioactive peak eluting at 19 minutes (Figure 2). Metabolite peaks eluting at approximately 10 and 17 minutes were substantially reduced as a result of the enzyme incubations. Metabolite fractions were collected from high-dose urine samples which were incubated overnight with glucuronidase/sulfatase enzymes. These HPLC-purified metabolites were submitted for mass spectral analysis.

Enzyme-hydrolyzed urine samples from the 1 mg/kg dosed rats or the dermal penetration group either contained less than detectable or minor concentrations of o-toluidine. The radioactivity profiles were similar for the two routes of exposure (Figures 3 and 4). The two major metabolites eluted at approximately 18 and 32 minutes and were subsequently identified as [CBI] and [CBI], respectively (Ref. 1). Structural representations of [CBI] are presented in Figure 5. A third metabolite eluting at approximately 13 minutes was analyzed by mass spectrometry but not structurally characterized.

Distribution of Radiolabelled Urinary Metabolites

The relative and absolute percent distributions for the major radioactive urinary metabolites are presented in Table 4. All urine samples were incubated overnight with glucuronidase/sulfatase enzymes. In the 0- to 12-hour urine samples from the 1 mg/kg dose group, [CBI] represented approximately 54 and 32 percent of the total urinary radioactivity, corresponding to approximately 33 and 19 percent of the total dose. Similarly, from dermally exposed rats [CBI] represented approximately 43 and 25 percent of urinary radioactivity in the 12-hour post-application urine sample corresponding to approximately 27 and 16 percent of the absorbed dose.

DISCUSSION

Dose dependent rate of urinary excretion was observed by comparing the percent of dose excreted in the 0- to 12-hour time interval between the 500 and 1 mg/kg dose groups. Approximately 60 percent of the 1 mg/kg dose was excreted in urine during the first 12 hours following dosing compared to 18 percent for the 500 mg/kg dose group. In addition, substantial amounts of unchanged [CBI] were detected in urine from 500 mg/kg group, whereas, only trace amounts were detected in urine from the 1 mg/kg group.

The 1 mg/kg oral dose level and the 1 mg dermal exposure were chosen because they represented potential low level workplace exposures. Approximately 60 percent of the administered oral dose and the systemically absorbed dermal exposure was excreted in the 0- to 12-hour post-dosing (application) sample. The urinary metabolite profiles (following glucuronidase/ sulfatase enzyme hydrolysis) were also very similar for the two exposure routes. The metabolite [CBI] represented approximately 33 percent of the administered oral dose and 27 percent of the absorbed dermal application in the 12-hour post-dosing (or application) urine sample. In the 12-hour urine samples, [CBI,] represented approximately 19 and 16 percent of the oral or absorbed dermal dose, respectively.

Based upon results from the study either [CBI] or [CBI] are good candidates for a urinary biological monitor to assess workplace exposure to [CBI] Results from the dermal exposure study indicated significant dermal penetration by liqui[CBI] Excretion data from both the dermal exposure and the 1 mg/kg oral dosing indicated rapid elimination in the first 12 hours following exposure. Therefore urine samples should be collected at the end of the work day in order to maximize the sensitivity of the urine monitoring procedure. In addition, collected urine samples should undergo incubations with glucuronidase/sulfatase enzymes in order to cleave any glucuronides or sulfate esters of [CBI] and [CBI]

REFERENCES

1. Williamson, J. A., Development of a Biomonitoring Assay for [CBI] or Its Metabolities in Human Urine, DuPont Haskell Laboratory, HLR 125-93. (1993)

TABLE 1

Urinary Excretion of Radioactivity Following
1 mg/kg or 500 mg/kg Oral Doses of [CBI]

Percent of Dose

1 mg/kg Dose

Timepoint (Hours)	Animal Number #1	#2	#3	#4	Ave ± S.D.
0-12	65.9	59.8	56.9	58.6	60.3 ± 3.9
12-24	6.8	8.0	10.1	9.4	8.6 ± 1.5
24-48	0.9	1.6	1.2	1.9	1.4 ± 0.4
Total Percent	73.6	69.4	68.2	69.9	70.2 ± 2.3

500 mg/kg Dose

Timepoint (Hours)	Animal Number #1	#2	#3	#4	Ave ± S.D.
0-12	16.8	15.4	17.6	23.3	18.3 ± 3.5
12-24	37.2	38.4	28.4	41.2	36.3 ± 5.5
24-48	18.0	11.5	19.8	6.9	14.1 ± 6.0
Total Percent	72.0	65.3	65.8	71.4	68.6 ± 3.6

TABLE 2

Disposition of Dermally Applied [CGI]

	Percent of Applied Dose			
	Animal			
Non-Absorbed Dose	#1	#2	#3	Ave ± S.D.
Cover Gauze plus Skin Wash	8.3	9.6	8.4	8.3 ± 0.7
Absorbed Dose				
Urine	9.7	12.8	10.2	10.9 ± 1.7
Feces	0.3	1.1	1.2	0.9 ± 0.5
Tissues and Carcass	0.8	1.1	0.8	0.9 ± 0.2
Cage Wash	0.9	1.5	1.8	1.4 ± 0.5
Total Percent Absorbed	11.7	16.5	14.0	14.1 ± 2.4
	Relative Percent Distribution of Absorbed Dose			
	Urinary Excretion			
Timepoint (Hours)	#1	#2	#3	Ave ± S.D.
0-12	69.2	58.2	60.7	62.7 ± 5.8
12-24	13.7	19.4	12.1	15.1 ± 3.8
Urine Total	82.9	77.6	72.8	77.8 ± 5.1
	Fecal Excretion			
Fecal Total (0-24 Hours)	2.6	6.7	8.6	6.0 ± 3.1
	Tissues and Carcasses			
24-Hour Sacrifice	7.7	6.7	5.7	6.7 ± 1.0

TABLE 3

Distribution of Radioactivity in Organs and Tissues Following Dermal Application of [CBI]

Organ or Tissue	µg Equivalents per Gram (mL) Tisssue* Animal #1	#2	#3	Ave ± S.D.
Blood	0.011	0.017	0.015	0.014 ± 0.033
Heart	0.003	0.008	0.007	0.006 ± 0.003
Lungs	0.013	0.026	0.032	0.024 ± 0.010
Liver	0.053	0.065	0.084	0.067 ± 0.016
Spleen	0.004	0.007	0.007	0.006 ± 0.002
Kidneys	0.031	0.041	0.046	0.039 ± 0.008
Testes	0.003	0.004	0.002	0.003 ± 0.001
Fat	0.007	0.011	0.011	0.010 ± 0.002
Brain	0.004	0.004	0.003	0.004 ± 0.001
Skin	0.18	0.12	0.07	0.12 ± 0.06
G.I.Tissue	0.075	0.049	0.049	0.058 ± 0.015
Plasma	0.006	0.008	0.005	0.006 ± 0.002

* µg Equivalent values were calculated based upon the specific radioactivity content of the [CBI] dose (21.6 µCi/mg).

TABLE 4

Distribution of Radioactive Urinary Metabolites Following Oral Dosing (1 mg/kg) or Dermal Application of [CSI]

Percent Values*

1 mg/kg Oral Dose - 12-Hour Urine

	Peak #1		4AC		NAAC	
Animal Number	Relative %	% of Dose	Relative %	% of Dose	Relative %	% of Dose
#1	14.3	9.4	31.5	18.8	54.2	35.7
#2	13.0	7.8	34.3	20.5	52.7	31.5
#3	14.2	8.1	30.5	17.4	55.3	32.4
#4	14.9	8.7	30.6	17.9	54.5	31.9
Average	14.1	8.5	31.7	18.7	54.2	32.9

Dermal Penetration - 12-Hour Urine

Animal Number						
#1	14.8	10.2	16.3	11.3	31.6	21.9
#2	22.7	13.2	27.3	15.9	50.1	29.2
#3	21.0	12.7	32.0	19.4	47.0	28.5
Average	19.5	12.0	25.2	15.5	42.9	26.5

* Relative percent values are the percent of radioactivity in an injected (HPLC) urine sample represented by each radioactive metabolite. The percent of dose values are based upon the administered dose for the oral gavage group, and the percent of absorbed dose for the dermal group.

FIGURE 1

HPLC/Ramona Chromatogram of [CBI]

HPLC Solvent Gradient Conditions For [CBI]

1) 100% 0.1 M Ammonium Acetate pH 3.3
Isocratically for 10 minutes

2) 50:50 Acetonitrile: 0.1 M Ammonium Acetate pH 3.3
25 minute linear gradient from Step 1

3) 50:50 Acetonitrile: 0.1 M Ammonium Acetate pH 3.3
Isocratically for 15 minutes

CBI

FIGURE 2

Representative HPLC/Ramona Profiles of Urine from the 500 mg/kg Dose Group. a) Non-incubated urine; b) Glucuronidase/Sulfatase Enzyme-Hydrolyzed Urine

a)

b)

FIGURE 3

Representative HPLC/Ramona Profile of a Glucuronidase/Sulfatase Hydrolyzed 12-Hour Urine Sample from 1 mg/kg Dosed Rats

FIGURE 4

Representative HPLC/Ramona Profile of a Glucuronidase/Sultatase Hydrolyzed 12-Hour Urine Sample from the Dermal Penetration Group of Rats

CERTIFICATE OF AUTHENTICITY

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